

In response to the observations made at Items 4 and 5 of the Official Action, previous claim 11 is cancelled herewith in favor of a new claim 35, which is claim 11 rewritten to remove the points of indefiniteness noted in the Official Action.

In response to the observations made at Items 6 and 7 of the Official Action, it is believed that the specification appropriately references the literature in this art, and does not refer to such literature for any "essential material". On the other hand, the Examiner's objection to literature references in the claims is well-taken, and claim 14 is appropriately amended to delete such references.

Concerning the drawing objection set at Item 8, the Form PTO-948 sets forth objections concerning margins and photograph quality. Those are the sort of objections that will of course be corrected upon allowance of the present application; however, it is not believed that prosecution is advanced by making proposed corrections of that sort at this time. Reconsideration is therefore respectfully requested, of the requirement at Item 8 to submit a proposed drawing correction in reply to the outstanding Official Action.

At Item 9a of the Official Action, the Examiner called for insertion of the appropriate sequence identification numbers into the specification, which has been done by the present amendment, at appropriate locations.

At Item 9b, a requirement was imposed to indicate any claim to benefit or priority in the specification.

Accordingly, page 1 of the specification is amended herewith to indicate that the present application is the U.S. national stage of the involved PCT International application. As the present declaration sets forth the ultimate priority claim to the original French application, all requirements as to claiming priority benefit are satisfied.

At Item 11 of the Official Action, claims 10-21, 27, 28 and 32-34, as previously in the case, were rejected under the first paragraph of 35 USC §112, as allegedly being based on an insufficient written description of the claimed invention, on the basis that the specification is not reasonably suggest that applicant was in possession of "any and all manner of variants [of GFP] that have the requisite properties." In a similar vein, at Item 12 of the Official Action, claims 10-21, 27 and 28 were further rejected under the first paragraph of 35 USC §112, as allegedly being based on a non-enabling disclosure. Those rejections are respectfully traversed, for the following reasons.

With respect to the written description rejection, applicants point out that the claims in question are original claims, and hence part of the written description of the invention. Therefore, as the original disclosure describes the invention in the identical terms used in the claims (by definition), the original disclosure provides strong evidence that the inventors had possession of the invention as claimed.

The current version of the USPTO written description guidelines for inventions in the biochemical arts does not require that applicants have actually reduced to practice all embodiments within the scope of a given claim, as such requirement would obviously be unreasonably burdensome to applicants, and would furthermore eliminate altogether the concept of a patent application serving as a constructive reduction to practice.

In support of the non-enablement rejection, page 6 of the Official Action sets forth a lengthy quotation from the *Genentech* case. However, that situation is plainly inapposite to the present specification, given that the ruling in *Genentech* is expressly predicated on a situation wherein there is "no disclosure of any specific starting material or any of the conditions under which a process can be carried out."

By contrast, in the present application, ample guidance and specific disclosure of the starting materials and conditions are set forth. Furthermore, applicants provide herewith four additional examples showing that the experimental conditions depend neither on the ligand/receptor complex nor is dependent on a unique donor/acceptor pair.

It is to be stressed that the present invention allows the detection of any target protein with any ligand.

These examples are presented here to show that applicants were in possession of several variants of GFP, for example EGFP, ECFP, EYFP, that have the requisite properties. They also show that the applicants were in possession of a

method that would allow for the detection and quantification of any ligand.

The above mentioned examples concern the following sets of receptor, ligand and fluorophores:

receptor	hM1 receptor	hM1 receptor	NK2R receptor	NK2R receptor	CXCR4 receptor
ligand	pirenzepine	pirenzepine	NKA	NKA	SDF-1 $\alpha$
fluorophores	EYFP- Bodipy 558/568	EGFP- Bodipy 558/568	ECFP- Texas red	EGFP- Texas red	EGFP- Texas red
Figure	19	19	20	21	22

### 1) General procedure for detection of interactions using different variants of the green fluorescent protein.

a) To meet the requirements of fluorescence resonance energy transfer, the energy acceptor absorption spectrum must overlap the energy donor emission spectrum. The fluorescent groups used are the following: bodipy 558/568 and Texas red, and they behave as energy acceptors for ECFP, EGFP and EYFP (used here as energy donors).

b) Detection of interactions is carried out by exciting the biological preparation with light at a wavelength appropriate for each donor (430 nm for ECFP, 470 nm for EGFP and EYFP), and by monitoring fluorescence at a wavelength appropriate for the detection of the fluorescence of each energy donor (480 nm for ECFP, 510 nm for EGFP and 530 nm for EYFP).

c) Using the set of excitation and emission wavelengths specific to each green fluorescent protein variant, the interaction with a fluorescent ligand is monitored as the decrease of donor fluorescence, due to energy transfer to the acceptor. This measurement is performed in exactly the same manner with all fluorophores listed in a). The detailed experimental protocol is given in example 5 part II of the present application.

## **ADDITIONAL EXAMPLES**

### **ADDITIONAL EXAMPLE 1 (Figure 19)**

#### **EGFP-hM1 or EYFP-hM1 interactions with pirenzepine bodipy.**

EGFP-hM1 and EYFP-hM1 constructs consist in fusions between the green and yellow variants of GFP, respectively, and the human muscarinic M1 receptor. Their preparation is described in example 7 of the present application.

The detection of the interactions between EGFP-hM1 or EYFP-hM1 with pirenzepine bodipy is described in Figure 19 A and B, respectively (see enclosures). In this example, bodipy corresponds to bodipy 558/568 and the ligand is pirenzepine.

Figure 19A illustrates emission fluorescence spectra of EGFP (closed circles) and EYFP (open circles) together with excitation (solid line) and emission (broken line) spectra of pirenzepine bodipy.

Figure 19B illustrates the diminution of EGFP fluorescence emission (EGFP-hM1 construct) at 510 nm (excitation at 470 nm) or of EYFP fluorescence emission (EYFP-hM1 construct) when pirenzepine bodipy is added to a suspension of cells expressing EGFP-hM1 or EYFP-hM1 receptors.

### **ADDITIONAL EXAMPLE 2 (Figure 20)**

#### **ECFP-NK2R interactions with NKA-Texas red.**

This additional example corresponds to example 5 of the present application which describes the detection of interactions between EGFP-NK2R and NKA-bodipy, and in this additional example, the fluorescent groups used here are ECFP and Texas red.

ECFP-NK2R is prepared as described in example 1 of the present application, using the cDNA encoding ECFP instead of that encoding EGFP.

Figure 20A shows excitation and emission spectra of HEK 293 cells expressing ECFP-NK2R (excitation: broken line and emission: solid line filled squares; maxima are at 430 nm and 480 nm, respectively) superimposed with excitation and emission spectra of Texas red (excitation -broken line- and emission -solid line filled circles; maxima are at 585 nm and 610 nm, respectively).

Figure 20B illustrates the diminution of ECFP fluorescence emission at 480 nm (excitation at 430 nm) when NKA-Texas red is added to a suspension of cells expressing ECFP-NK2R.

**ADDITIONAL EXAMPLE 3 (Figure 21)**  
**EGFP-NK2R interactions with NKA-Texas red.**

This additional example corresponds to example 5 of the present application which describes detection of interactions between EGFP-NK2R and NKA-bodipy, and in this additional example, the fluorescent groups used here are EGFP and Texas red.

Fig 21A shows excitation and emission spectra of HEK 293 cells expressing EGFP-NK2R and of NKA-Texas red. Excitation and emission maxima are at 470 nm and 510 nm for EGFP and at 585 nm and 610 nm for Texas red.

Figure 21B illustrates the diminution of EGFP fluorescence emission at 510 nm (excitation at 470 nm) when NKA-Texas red is added (at the indicated concentrations, in nM) to a suspension of cells expressing EGFP-NK2R.

**ADDITIONAL EXAMPLE 4 (Figure 22)**  
**EGFP-CXCR4 interactions with SDF-1 $\alpha$ -Texas red.**

This additional example corresponds to example 9 of the present application describing preparation of a fluorescent CCR5 receptor. CXCR4, like CCR5 (in example 9 of the present patent application) is a chemokine receptor. The natural ligand of CXCR4 is the chemokine SDF-1 $\alpha$  (Stromal Derived Factor 1 $\alpha$ ). The fluorescent CXCR4 receptor is obtained as described in examples 1, 7 and 9 by fusing, in frame, the gene encoding EGFP to the 3' end of the gene encoding the CXCR4 receptor. The fluorescent chemokine SDF-1 $\alpha$  is obtained by chemically labeling Lys 68 of the chemokine with Texas red-NHS (N-hydroxysuccinimide).

The determination of the interactions between fluorescent CXCR4 and fluorescent SDF-1 $\alpha$  is performed as described in example 1 part II of the present application by setting excitation and emission wavelengths to EGFP detection (470 nm for excitation and 510 nm for detection).

Figure 22A shows the diminution of EGFP fluorescence emission at 510 nm (excitation at 470 nm) when SDF-1 $\alpha$  - Texas red is added (at the indicated concentrations, in nM) to a suspension of cells expressing EGFP-CXCR4.

Figure 22B shows the reversion of SDF-1 $\alpha$  - Texas red binding (at the indicated concentrations) by addition of 500 nM unlabelled SDF-1 $\alpha$ .

A detailed description of the experimental protocol and uses of this detection procedure can be found in Valenzuela, Palanche et al. 2001 (accompanying paper).

In light of the above discussion, therefore, it is believed to be apparent that both the written description and non-enablement rejection should be withdrawn and not repeated with respect to the claims as they now appear in the case.

At pages 7 and 8 of the Official Action, the claims previously in the case were also rejected as being indefinite.

Many of the bases for the indefiniteness rejection were considered to be well-taken, and the Examiner will observe in claims 10, 14 and 32-34 as amended herewith, as well as in the new claim 35, that those bases for indefiniteness have been addressed and corrected. Favorable reconsideration and withdrawal of that rejection are therefore respectfully requested.

As no cited reference was applied against any of the claims, it is further believed that the claims are considered to be free of the prior art of record.

In view of the present amendment and the foregoing remarks, therefore, it is believed that this application has been placed in condition for allowance, with the remaining

GALZI et al. S.N. 09/445,205

claims 10, 12-21 and 32-35, as amended. Allowance and passage to issue on that basis are accordingly respectfully requested.

Respectfully submitted,

YOUNG & THOMPSON

By *Amik Castel*  
*Res. No. 35,041 for*  
Andrew J. Patch  
Attorney for Applicants  
Registration No. 32,925  
745 South 23rd Street  
Arlington, VA 22202  
Telephone: 703/521-2297

July 17, 2001



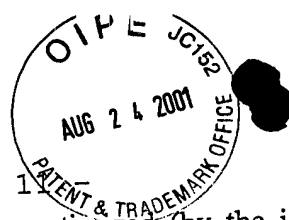


**RECEIVED**

AUG 28 2001

TECH CENTER 1600/2900

VERSION WITH MARKINGS TO SHOW CHANGES MADE



RECEIVED

AUG 28 2001

TECH CENTER 1600/2900

- 1 The various GFP mutants can also be optimized (by the introduction of silent mutations which optimize the use of codons specific to each species) for expression in the following cells:

- human: ref. Haas et al. 1996 Curr. Biol. 6:315-323; Yan et al. 1996, Nucleic Ac. Res. 24:4592-4593; Zolotukhin et al. 1996, J. Virol. 70:4646-4654
- bacterial: Cramer et al. 1996 Nature Biotechnol. 14:315-319, Cormack et al. 1996, Gene, 173:33-38 for *Escherichia coli*,
- plant: Reichel et al. 1996, Proc. Natl. Acad. Sci. 93:5888-5893.

10

. GFP :

represented by the sequence  
SEQ ID NO: 2

SEQ ID NO: 1

The term GFP indicates a protein coded for by the nucleotide sequence given in Figure 1, and which emits a fluorescence once it is expressed in cells. GFPs with substitutions, additions or deletions of amino acids which have an influence either on the fluorescence properties or on the degree of expression of the GFP are referred to as GFP mutants.

15

The main characteristics of the fluorescent proteins advantageously used in the process of the invention are given below:

Protein	maximum $\lambda$ -excitation	$\lambda$ -emission	extinction coefficient	quantic yield
EYFP	514	527	36500	0.63
ECFP	432	480	18000	0.67
GFPUV	395	509	21000	0.77
EGFP	489	511	39000	0.66

20

The autofluorescent protein BFP is preferably excluded since it does not satisfy the conditions defined for the autofluorescent proteins of cnidarians, namely a molar extinction coefficient greater than  $14000 \text{ M}^{-1}\text{cm}^{-1}$  and a quantic fluorescence yield greater than 0.38.

25

The invention also relates to the use of a fluorescent protein (No 1) as defined above, in which the ligand is labeled

- \* either with a fluorescent substance, the labeling being carried out:

- either via a chemical route, the fluorescent substance then being a

30

chemical compound,

- or or via a recombinant route, the fluorescent substance then being a fluorescent peptide or protein (No 2) which can be chosen in particular from the fluorescent proteins obtained or derived from autofluorescent proteins of

annually updated and edited list as a supplement under the name: "Receptor and Ion Channel Nomenclature" by Elsevier Trends journals, in Trends in Pharmacological Sciences.

5 3) nuclear receptors containing a domain for interaction with DNA which are structurally linked to the steroid receptor (Mangelsdorf et al. 1995, Cell, 83:835-839, Wurtz, J.L. et al. 1996, Nature Struct. Biol. 3:206).

4) plasmid membrane receptors with tyrosine kinase activity, which are structurally linked to the insulin receptor (Yarden, Y. and Ullrich, A. 1988, Biochemistry 27:3113-3119).

10 5) membrane-bound receptors coupled to the tyrosine kinase proteins (STATs, TYK2, Jak) which are structurally linked to the  $\gamma$  interferon receptor (Brisco, J. et al. 1996, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 351:167-171; Ihle, J.N. 1995, Nature 377:591-594).

15 When the fusion is carried out between EGFP and a receptor coupled to the G proteins (group 1), the fusion can be carried out in particular:

1) on the N-terminal side of the receptor, and thus on the C-terminal side of EGFP,

20 2) on the C-terminal side of the receptor and thus on the N-terminal side of EGFP,

3) in the receptor sequence, in particular in the first or third intracellular loop, optionally by introducing one or more copies of a spacer sequence, in particular -GGGGS-, represented by the nucleotide sequence SEQ ID NO: 3.

25 When the fusion is carried out between EGFP and a receptor-channel (group 2), the fusion can be carried out in particular:

1) in the region homologous to the "major immunogenic region" of the  $\alpha$  sub-unit of the nicotinic receptor of Torpedo (residues 67-76), optionally by introducing one or more copies of a spacer sequence, in particular -GGGGS-, represented by the nucleotide sequence SEQ ID NO: 3.

30 When the fusion is carried out between EGFP and a nuclear receptor (group 3), the fusion can be carried out in particular:

1) on the N-terminal side of the receptor, and thus on the C-terminal side of EGFP,

35 2) on the N-terminal side of the receptor, truncated in its N-terminal part upstream of the DNA binding domain, and thus on the C-terminal side of EGFP.

Examples of insect cells:

Mention may be made of *Lepidoptera* lines e.g. *Spodoptera frugiperda* (Sf9) or *Trichoplusia ni* (Tni). The transformation methods (in particular infection) are described in Current Protocols in Molecular Biology (op.cit.).

5

## THE LIGANDS

10 The ligands which interact with the target protein can be of any origin (natural, synthetic, semi-synthetic or recombinant) and of any structure (chemical, peptide or protein). They can be naturally fluorescent (or a bearing a chromophore) or can either require a chemical reaction for grafting a fluorescent group (or a precursor of a fluorescent group) or a chromophore, or require a DNA construct leading to fusion of the ligand with GFP and allowing the expression of the ligand thus made fluorescent.

15

Examples of chemical reactions are:

- the coupling of amines or thiols with reagents such as alkyl halides, aryl halides, acyl halides, acid halides, the isothiocyanate group, the maleimide group or epoxides, in an organic solvent in the presence of a base or in aqueous medium,
- 20 - coupling of acids with amines activated with groups such as succinimides.

25 According to the process of the invention, the fluorescence of the transformed cells can be measured in a spectrofluorimeter with the aid of which the spectral properties of the cells, in suspension or adherent, can be determined by acquisition of their excitation and emission spectra. The interactions with the fluorescent ligand are then detected by means of the changes in the donor's and acceptor's excitation and/or emission energy spectra, and the ligands are defined as being pharmacologically significant if their interactions with the target protein are  
30 inhibited by the addition of an excess of non-fluorescent ligand which prevents the interaction between the fluorescent target protein and the fluorescent ligand.

## DESCRIPTION OF THE FIGURES :

35

- Figure 1 gives the nucleotide sequence coding for the wild-type GFP (Prasher et al. 1992, Gene 111:229-233) of *Aequorea victoria*.

(SEQ ID NO: 1)

represented by  
proteinic seq  
SEQ ID NO: 2

- Figure 2 represents the fluorescence measured during the expression of the construct pMT3-EGFP-C3-SP. The fluorescence expressed in counts per

- Figure 5b represents the binding of the antagonist  $^3\text{H}$  SR 48968 to cells expressing the fluorescent receptor NK2R-RF1. The amount of  $^3\text{H}$  SR 48968 bound is given on the y-axis (in disintegrations per minute; dpm) and the concentration of  $^3\text{H}$  SR 48968 added to the sample is given on the x-axis. The gray and black diamonds represent the total binding of  $^3\text{H}$  SR 48968 in two different experiments, and the gray crosses and squares represent the non-specific binding determined in the presence of an excess of neurokinin A (10  $\mu\text{M}$ ). The lines correspond to the theoretical curves for the binding of a ligand to its receptor or to non-specific binding sites. The affinity values determined (KD) are 0.8 nM and 0.92 nM in each of the two experiments. The maximum binding (B max) values are, respectively, 0.075 and 0.088 pMol/25,000 cells.

- Figure 6a represents the response functionality test for the release of intracellular calcium (FURA 2) for cells expressing the DNA construct pCEP4-NK2R WT. The fluorescence at 510 nm is given on the y-axis (expressed in counts per second), the excitation taking place at 340 nm, and the time (in seconds) is given on the x-axis. The responses are elicited by the agonist neurokinin A (NKA) and inhibited by the antagonist cyclo(-Gln-Trp-Phe-Gly-Leu-Met) (cyclopeptide). In experiment 1, 10 nM NKA are added. In experiment (2, 3), 5  $\mu\text{M}$  of cyclopeptide (2), and then 10 nM of NKA (3) are successively added.  
Ⓢ represented by the sequence SEQ ID NO : 4.

- Figure 6b represents the response functionality test for the release of intracellular calcium (FURA 2) for cells expressing the DNA construct pCEP4-NK2R-RF1. The fluorescence at 510 nm is given on the y-axis (expressed in counts per second), the excitation taking place at 340 nm, and the time (in seconds) is given on the x-axis. The responses are elicited by the agonist neurokinin A (NKA) and inhibited by the antagonist cyclo(-Gln-Trp-Phe-Gly-Leu-Met) (cyclopeptide). In experiment 1, 10 nM NKA are added. In experiment (2, 3), 5  $\mu\text{M}$  of cyclopeptide (2), and then 10 nM of NKA (3) are successively added.

- Figure 7 represents the purification of the peptide NKA BO I by reverse-phase HPLC. The time (in minutes) is given on the x-axis and the optical density (mV) is given on the y-axis. The detection is carried out at two wavelengths: 219 nm (broken lines) and 530 nm (solid lines).

The identical peaks 1, 2 and 3 are, respectively, NKA, the NKA-Bodipy derivative and the Bodipy-IA reagent.

containing the GFP are revealed by chemoluminescence using the ECL Kit available from Amersham.

5 The figure reveals the presence of EGFP-RANTES in lanes 3 and 6 (yeast culture supernatant and yeasts, respectively) expressing the DNA construct EGFP-RANTES (42,000 dalton band). Lanes 1 and 8 correspond to the migration of 20-, 30-, 40- and 50-kilodalton annotated molecular weight markers. Lane 2 corresponds to the control yeast supernatant (trypsinogen), lane 4 corresponds to the EGFP-RANTES dialysis precipitate, lane 5 corresponds to the control yeast  
10 and lane 7 corresponds to the yeast expressing EGFP alone.

### EXAMPLES

#### 15 EXAMPLE 1: DNA CONSTRUCTS COMPRISING A FUSION BETWEEN EGFP AND THE AMINO-TERMINAL END OF THE NK2R RECEPTOR OF TACHYKININS

##### I) Fusion of EGFP with a signal peptide:

20 The cDNA coding for EGFP (Figure 1) is fused in phase with the sequence coding for the signal peptide of the hen alpha 7 sub-unit (Genbank Accession No: X522995) coding for an acetylcholine nicotinic receptor as follows:

A restriction site for the endonuclease BsrG I is introduced onto the coding  
25 codons 1 to 9 of EGFP using the oligonucleotide <sup>Ⓢ represent by the nucleotide sequence SEQ ID NO</sup> 5'GGTCGCCACCCTGTACAAGAAGGGCGAGG3', reagents provided in the mutagenesis kit RPN 1526 (Sculptor) supplied by the Amersham company, and single-stranded pEGFP C3 prepared from the plasmid pEGFP C3 (Genbank Accession No US 57607) supplied by the ClonTech company. The mutant pEGFP  
30 C3-1 obtained is sequenced and then cloned in phase with the signal peptide of alpha 7 by ligation of two fragments: the 5225-nt fragment BsrGI-Xho of pJL223 (Eiselé et al. 1993, Nature 366:479-483) and the 725-nt fragment BsrG I - Xho I of pEGFP C3-1. The plasmid pJL223 contains the gene for the protein a7-V201-5HT3 between the sites Not I and Xho I of the vector pMT3 (Swick, A.G. et al.  
35 1992, Proc. Natl. Acad. Sci. 89:1812-1816). The construct obtained, named pMT3-EGFP-C3-SP, is transiently late expressed in HEK 293 cells (ATCC CRL 1573) after transfection with calcium phosphate (Cheng and Okayama 1986), in order to check that the construct is correct. The fluorescent emission spectrum (excitation at 450 nM) of the culture supernatants of cells expressing pMT3-EGFP

C3-SP or of and transfected cells (concentrated five-fold by centrifugation on centrikon 10 Amicon)) are recorded. In Figure 2, which shows the difference between the spectrum of transfected and non-transfected cells, the emission peak of EGFP is clearly seen, which indicates that the construct does indeed lead to the expression of EGFP secreted into the culture medium.

II) Cloning of the receptor NK2R of tachykinins into the mutagenesis vector KS and the expression vector pCEP4:

The 2997-nt fragment Spe I - Hind III from the plasmid prTKR1-1 (Pr. S. Nakanishi, Kyoto university, Japan, Biochem. Biophys. Res. Comm. 1989, 165:695-702), containing the cDNA coding for the rat NK2R receptor (Genbank Accession No: M31838), is ligated with the 3549-nt fragment Spe I - Hind III from the vector pBluescript KS (+) to give the plasmid pKS NK2R.

The 1369-nt fragment Not I - BsrG I from the plasmid prTKR1-1 is ligated with the 1663-nt fragment BsrG I - Xho I from pKS NK2R and the 10370-nt fragment Not I - Xho I from pCEP4 to give the plasmid pCEP4-NK2R.

III) Fusion of EGFP between the signal peptide of alpha7 and the amino-terminal end of the tachykinin NK2R receptor :

The 816-nt fragment Not I - Xho I from pMT3-EGFP C3-SP is ligated with the 12856-nt fragment Not I Xho I (Xho I partial digestion) from pCEP4-NK2R to give the construct pCEP4-NK2R-RF1 (Figure 3).

## EXAMPLE 2 : CONSTRUCTION OF DNA COMPRISING THE FUSION OF GFP INTO THE INTRACELLULAR LOOPS 11 AND 13 OF THE TACHYKININ NK2R RECEPTOR

30

I) Introduction of the EGFP cloning sites into the loop i1 or i3 of the NK2R receptor:

The single-stranded DNA of pKS NK2R is mutagenized as in 1a) with the oligonucleotides

i1: 5'CACGAGAGGATGTACAACCTCGAGCGCACAGTCACC3' containing the mutations for the cloning sites BsrG I and Xho I, allowing the introduction of EGFP between amino acids 65 and 66, and

⊗ represented by the nucleotide sequence SEQ ID NO: 6,

i3: 5'GTACCCAGACACCAGCTAGCAGATCTGAAGCTTCGCCATCAGGC3' containing the mutations for the cloning sites Nhe I, Bgl II and Hind III allowing the introduction of EGFP between residues 233 and 234 or 233 and 238.

- 5 The plasmids obtained pKS NK2R-i1 and -i3 are introduced into competent XL1Blue bacteria (transformation), and the plasmid DNA samples isolated from the ampicillin-resistant colonies are screened for the presence of the sites introduced, respectively, by the mutations with the oligonucleotides i1 and i3.

10 II) Cloning of EGFP in the loops i1 and i3 of the tachykinin NK2R receptor:

- cloning into the loop i1: the fragments Hind III - BsrG I, and Hind III - Xho I, of pKS NK2R-i1 are ligated with the fragment BsrG I - Xho I, 725 nt, of pEGFP C3-1. The insert of 3741 nt coding for the fusion protein is then excized with the enzymes Spe I and Sal I and ligated with the vector pCEP4 opened by the enzymes Nhe I and Xho I to give the DNA construct pCEP4 NK2R-RF2 ;

- cloning into the loop i3: two constructs are obtained:

- the 744-nt fragments Nhe I - Bgl II of pEGFP C3 are ligated with the fragments Not I - Nhe I and Bgl II - Not I of pKS-NK2R-i3, the 3750-nt insert Spe I - Sal I obtained is then cloned into the vector pCEP4 between the sites Nhe I and Xho I to give the DNA construct pCEP4-NK2R-RF3.

- the 757-nt fragments Nhe I - Hind III of pEGFP C3 are ligated with the fragments Not I - Nhe I and Hind III - Not I of pKS-NK2R-i3, the 3770-nt insert Spe I - Sal I thus obtained is then cloned into the vector pCEP4 between the sites Nhe I and Xho I to give the DNA construct pCEP4-NK2R-RF4.

EXAMPLE 3 : EXPRESSION OF RECOMBINANT PROTEINS AND FUNCTIONAL CHARACTERIZATION

I) Expression :

- HEK 293 cells are transfected, by the preparation method with calcium phosphate (Chen & Okayama 1987, Mol. Cell. Biol. 7:2745-2752, Current Protocols in Molecular Biology, op.cit.), with the DNA constructs pCEP4-NK2R-RF1, -RF2, -RF3 and -RF4 stable lines are established by selecting transfected cells resistant to hygromycin (100 µg/ml, Clontech). The cells are cultured in the presence of 100 µg/ml hygromycin in MEM medium (Gibco) supplemented with 10% fetal calf serum (Seromed),



Figure 14 shows that the amplitude of the fluorescence signal measured at 510 nm (excitation at 460 nm) decreases when the concentration of non-fluorescent molecule present in the test increases. The curve for the inhibition of the binding  
5 of NKA Bo I makes it possible to estimate the affinity of the non-fluorescent neurokinin A and SR48968 molecules.

Figure 15 corresponds to the experiment described in Figure 14, carried out in the presence of neurokinin A (NKA) or SR48968.

10

EXAMPLE 6 : CLONING OF THE NK2R-RF1 CDNA INTO THE VECTOR PPIC9 AND EXPRESSION IN THE YEAST *PICHLA PASTORIS*

a) Cloning :

15 The portion of pCEP4-NK2R-RF1 coding for the fusion protein is amplified by PCR (Current Protocols in Molecular Biology, op.cit.) using the primers 5'GGAGAGTTCCAACTCGAGAAAAGAAAGAAGGGCGAGGAG3' and 5'GTCAGCTGTTTCTGCGGCGCGCTAAGCCTGGGCCTT3', ⊕  
allowing 1) the production of 1868-nt fragment coding for all of the fusion protein  
20 NF2R-EGFP except for the signal peptide, and 2) the in-phase cloning into the expression vector of yeast pPIC9 (Invitrogen) with the sequence coding for the signal peptide of the promoter factor alpha of the gene AOX1. The cloning sites used are, respectively, XhoI for the 5' end of the amplification product and Not I for its 3' end.

25

b) Expression :

The yeasts are transformed with the linearized pPIC9-NK2R-RF1 plasmid (StuI or SalI) and are cultured on histidine-free MD culture medium, prepared according to the instructions in the manual supplied with the vector pPIC9 (inVitrogen). The  
30 expression of the DNA construct introduced into the cells is induced with methanol. For this, the colonies are propagated in liquid medium (BMGY) for 24 h, and are then transferred into BMMY medium containing 0.5% methanol, allowing induction of the expression of the DNA construct NK2R-RF1. Aliquots of these cultures are withdrawn and the clones expressing the fluorescent protein  
35 identified by measuring the excitation and emission spectra of EGFP.

EXAMPLE 7 : CONSTRUCTION OF DNA CODING FOR THE MUSCARINIC RECEPTOR OF ACETYLCHOLINE FUSED WITH EGFP AND EXPRESSION IN MAMMALIAN CELLS.

- 5 I) Cloning : the cDNA fragment coding for the human muscarinic receptor M1 (Genbank Accession No X15263) is amplified by PCR (Current Protocols in Molecular Biology, op.cit.) using the primers:

5'TTAGTTCTAAACTAGCGGCCGCACTAGTCCTCCATGAACACTTC  
AGCCCCA3' and

- 10 5'CTTGAACCTATAGCTAGCCTCGAGTCAGCATTGGCGGGAGGG3',  
*represented respectively by the nucleotide sequences SEQ ID NO: 10 and  
SEQ ID NO: 11.*

- The 1383-nt fragment obtained is cleaved with the enzymes Not I (at the 5' end)  
and Xho I (at the 3' end) and ligated with the KS vector opened with the same  
enzymes (2888-nt fragment) to give the construct KS-hM1, or ligated with the  
15 vector pCEP4 opened with the same enzymes to give the construct pCEP4-hM1.

The construct KS-hM1 is used for the production of single-stranded DNA  
(Current Protocols in Molecular Biology, op.cit.) and the production of mutants.

- 20 II) Fusion of EGFP at the N-terminal of the hM1 receptor.

The oligonucleotide 5'CCTGCTGTCTCAGATCTCATCACCGTCC3' <sup>⊗</sup> is used,  
together with the reagents in the Sculptor mutagenesis kit (Amersham), to produce  
a mutant which allows the fusion in position 13 of the sequence coding for the  
hM1 receptor by means of the introduction of a restriction site for the enzyme Bgl

- 25 II. <sup>⊗</sup> *represented by the nucleotide sequence SEQ ID NO: 12,*

- The mutant obtained is digested with the enzymes Bgl II and Xho I and the  
1354-nt fragment generated is ligated with the 812-nt Not I - Bgl II fragment of  
pCEP4-NK2R-RF1, and the vector pCEP4 opened with the enzymes Not I and  
30 Xho I, to give the DNA construct pCEP4-hM1-RF1.

III) Expression of the fusion protein and hM1-RF1.

- The plasmid pCEP4-hM1-RF1 is introduced into HEK 293 cells by transfection  
with calcium phosphate or into Cos 1 cells by electroporation (Current Protocols  
35 in Molecular Biology, op.cit.).

The expression of the protein is detected as described above.

#### IV) Synthesis and purification of the muscarinic ligand ABT-Bodipy.

The base ABT (3-[2'-aminobenzhydryloxy]tropane) is dissolved in DMF (10 mM). 20 µl of this solution (0.2 µMol) are mixed with 4 µl of a 100 mM solution of Bodipy -IA and left at room temperature for 20 h. The reaction product is purified by HPLC (Gilson) on a Z5C8 25F reverse-phase column (Zorbax) on which is developed a linear gradient of from 10 to 95% solvent B over 60 minutes (A: H<sub>2</sub>O 0.1% TFA; B: CH<sub>3</sub>CN 0.1% TFA) with a flow rate of 1 ml/min. The detection wavelengths are set at 219 nm (peptide) and 530 nm (fluorophore). The product eluted (ABT Bo) at time 34 min. (Figure 7) is collected, concentrated by evaporation and re-suspended in DMF.

#### V) Fusion of EYFP at the N-terminal of the hM1 receptor and detection of the interaction with pirenzepine-bodipy 558/568.

This construct is prepared in an identical manner to that for the construct described in point II.

The interactions of the fusion protein with its ligand are effected using the ligand pirenzepine-bodipy in which the fluorescent group corresponds to bodipy 558/568 (4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid).

#### EXAMPLE 8 : DNA CONSTRUCT CODING FOR FLUORESCENT NICOTINIC RECEPTORS

a) DNA construct coding for the fusion of EGFP in the MIR region of the a7-V201-5HT<sub>3</sub> receptor.

The plasmid pJL223 (Eiselé et al. 1993, Nature 366:479-483) contains the gene for the protein a7-V201-5HT<sub>3</sub> which forms a receptor channel that is activated by acetylcholine and nicotine during its expression in *Xenopus* oocytes. The coding cDNA is between the sites Not I and Xho I of the vector pMT3 (Swick, A.G. et al. 1992, Proc. Natl. Acad. Sci. 89:1812-1816).

The 1424-nt insert Not I - Xho I is cloned between the sites Not I and Xho I of the Bluescript vector and the plasmid obtained (KS 223) serves as a matrix for the production of single-stranded DNA.

The oligonucleotide

5'CAGATCATTAGTTGTACAGGAAAGATCTTGAGGATCCTGGAGTGAAG3'  
represented by the nucleotide sequence SEQ ID NO: 13;

is used to introduce, on KS223, the restriction sites for the enzymes BsrG I, Bgl II and BamH I in the same phase as that of the identical sites borne by the plasmid pEGFP-C3. The mutation is introduced into a region of the receptor known as the MIR (Major Immunogenic Region, Barkas *et al.* 1987, Science, 235:77-80) between amino acids 63 and 64.

The fragments Not I-BsrG I (267 nt) and Bgl II-Xho I (1147 nt) of this mutant of KS 223 are ligated with the fragment BsrG I-Bgl II (721 nt) of pEGFP-C3 and the vector pCEP4 opened by the enzymes Not I and Xho I, to give the DNA construct pCEP4-223-RF1.

The fragments Not I-BsrG I (267 nt) and BamH I-Xho I (1138 nt) of this mutant of KS 223 are ligated with the fragment BsrG I-BamH I (772 nt) of pEGFP-C3 and the vector pCEP4 opened by the enzymes Not I and Xho I, to give the DNA construct pCEP4-223-RF2.

b) Construction of DNA coding for the fusion of EGFP in the cytoplasmic region of the receptor  $\alpha 7$ -V201-5HT<sub>3</sub>.

The cDNA coding for the protein  $\alpha 7$ -V201-5HT<sub>3</sub> contains in its cytoplasmic domain the sequences of the sites Avr II and Pst I, respectively, in phase and cohesive with the sequences cleaved by the enzymes Nhe I and Pst I of the plasmid pEGFP-C2 (ClonTech) and allow the production of the fusion protein containing the sequence of EGFP in the cytoplasmic domain of the protein  $\alpha 7$ -V201-5HT<sub>3</sub>.

The DNA construct coding for this fusion protein is thus obtained by ligating the fragments Not I-Avr II (1036 nt) and Pst I - Xho I (286 nt) of KS 223 with the fragment Nhe I - Pst I (774 nt) of pEGFP-C2 and the vector pCEP4 opened by the enzymes Not I and Xho I to give the construct pCEP4-223-RF3.

The constructs pCEP4-223-RF1, -RF2, and -RF3 are then expressed in HEK 293 cells as defined above.

#### EXAMPLE 9 : DNA CONSTRUCT CODING FOR A FLUORESCENT CHEMOKINE RECEPTOR

The cDNA coding for human chemokine receptor CCR5 (Genbank access No: U54994) is amplified using the oligonucleotides 5'GGCCCAAGCTTATGTCAGGATCCGGGGAT3', represented by the nucleotide sequence SEQ ID NO:14, and

5'CGCCCGCTCGAGTCACAAGCCCACAGATAT and then cloned into the vector Bluescript KS opened with the restriction enzyme Eco RV.

5 The insert is excized with the enzymes Hind III or BamH1 (5') and Xho I (3') (fragment 1) in order to be cloned in phase with the cDNA coding for EGFP or ECFP.

10 The in-phase fusion of EGFP or ECFP with the signal peptide of the plasmid pJL223 (Example 1) and the gene coding for the receptor CCR5, is carried out in the following way. The plasmid pJL223 is opened with the restriction enzyme Age I and then made blunt by mixing with Klenow and nucleotides. The fragment extending as far as the Xho I site is then eliminated (cleavage with Xho I) (fragment 2).

15 The plasmids pEGFP-C3 and pECFP-C3 are opened with the enzyme Age I and the ends are made blunt. The inserts Age I blunt- Hind III and Age I blunt- Xho I (fragments 3) are ligated with fragments 1 and 2 to give rise to the constructs KS-SP EGFP-Hind III-CCR5, KS-SP EGFP-Xho I-CCR5, KS-SP ECFP-Hind III-CCR5 and KS-SP ECFP-Xho I-CCR5. The cDNAs corresponding to the  
20 sequences SP EGFP-Hind III-CCR5, SP EGFP-Xho I-CCR5, SP ECFP-Hind III-CCR5, and SP ECFP-Xho I-CCR5 are excised with Not I and Xho I and then cloned into the vector pCEP4 opened with the same enzymes.

25 Figure 16 shows the emission spectra of transfected HEK 293 cells expressing the receptor EGFP-Hind III-CCR5, and Figure 17 shows the intracellular calcium release response of cells expressing the DNA construct EGFP-HindCCR5.

#### EXAMPLE 10 : DNA CONSTRUCT CODING FOR THE FLUORESCENT HUMAN CHEMOKINE RANTES

30

The cDNA coding for the human chemokine RANTES (Genbank access number M21121) is synthesized by recursive PCR using the oligonucleotides

35

- 1 5'GTTGACAAGCTTCGGGATCCA3' represented by the nucleotide sequence  
SEQ ID NO: 16,
- 2 5'AGCACAGAGGGCAGTAGCAATGAGGATGACAGCG  
AGGCGTGCCGCGGAGACCTTCATTGGATCCCGAAGCTTGTCAAC3'  
represented by the nucleotide sequence SEQ ID NO: 17,

- 3 5'ATTGCTACTGCCCTCTGTGCTCCTGCATCTGCCTCC  
CCATATTCCTCGGACACCACACCATGCTGCTTCGCCTACATT3',  
represented by the nucleotide sequence SEQ ID NO: 18,
- 4 5'GCACTTGCCACTGGTGTAGAAATACTCCTTGATGTGG  
5 GCACGGGGCAGTGGGCGGGCAATGTAGGCGAAGCAGCATGG3',  
represented by the nucleotide sequence SEQ ID NO: 19,
- 5 5'GCACTTGCCACTGGTGTAGAAATACTCCTTGATGT  
GGGCACGGGGCAGTGGGCGGGCAATGTAGGCGAAGCAGCATG  
G3', represented by the nucleotide sequence SEQ ID NO: 20,
- 10 6 5'CTAGCTCATCTCCAGCGAGTTGATGTACTCCCGAACC  
CATTCTTCTCTGGGTTGGCACAAACTTGACG3',  
represented by the nucleotide sequence SEQ ID NO: 21,
- 7 5'AACTCGCTGGAGATGAGCTAGGCGGCCGCTCG  
15 AGGTCGACCTAGTCACTA3', represented by the nucleotide sequence  
SEQ ID NO: 22,
- 8 5'TAGTGACTAGGTCGACCTCGA3', represented by the nucleotide  
sequence SEQ ID NO: 23,

according to the protocols described in Prodromou & Pearl, Protein Engineering  
20 Vol 5, pp 827-829, 1992, and then cloned into the vector Bluescript KS opened  
with Eco RV.

The DNAs coding, respectively, for RANTES chemokine (fragment Hind III -  
Not I or Bam HI-Not I) and for yellow fluorescent protein (fragments Age I blunt-  
25 Bam HI or Age I blunt- Hin DIII of the plasmid pEYFP-C3) are cloned in phase  
with the signal peptide of the alpha-mating factor of yeast between the sites Sna  
BI and Not I of the vector pPIC 9, to give the constructs pPIC9-EYFP- HindIII-  
RANTES and pPIC9-EYFP-Bam HI-RANTES.

30 Figure 18 shows an immunoblot revealing the expression of the construct EYFP-  
HindIII-RANTES in culture supernatant from yeast *pichia pastoris* transformed  
with the plasmid pPIC9-EYFP- HindIII-RANTES and detected using an anti-GFP  
antibody.

### 35 EXAMPLE 11 : IDENTIFICATION OF NOVEL LIGANDS FOR ORPHAN RECEPTORS

1) Fusion of EGFP with the carboxy-terminal end of the tachykinin NK2R  
receptor.

The cDNA coding for the NK2R receptor included in the plasmid pKS NK2R (Example 1) is cleaved with the enzyme Age I. The protruding 5' end obtained is made blunt with the aid of nucleotides and Klenow polymerase. The linearized DNA is cleaved with the enzyme Not I to produce a 1045-nt fragment (fragment

5 a).

The plasmid pEGFP-C3 is opened with the aid of the enzyme Age I and then made blunt. It is then cleaved with the enzyme Xho I to give a 739-nt fragment, b.

10 Fragments a and b are ligated with the vector pCEP4 opened with the enzymes Not I and Xho I to give the plasmid pCEP4-NK2R-AgeI-EGFP.

2) Fusion of the fluorescent protein EYFP with the amino-terminal of the alpha sub-unit of the mouse protein Gq

15 The cDNA coding for the alpha sub-unit of the mouse protein Gq (Genbank Accession No M55412) is amplified by PCR using the oligonucleotides 5'GCGGCCGCATGGGGGATCCTACTCTGGAGTCCATCATGGCG and 5'CCGCTCGAGTTAATCTAGAAGGACCAGATTGTACTCCTTCAGG in order to introduce Not I and Bam HI sites at the 5' end and Xho I and Xba I at the

20 3' end of the gene coding for the alpha sub-unit of the protein Gq.

⊗ represented respectively by the nucleotide sequences SEQ ID NO: 24 and SEQ ID NO: 25,

The PCR product obtained is cloned into the vector KS opened with Eco RV.

25 The cDNA coding for the alpha sub-unit Gq is excised with the enzymes BamHI and Xba I and ligated in the plasmid pEYFP-C3 opened with the enzymes Bgl II and Xba I to give the plasmid pEYFP-Bgl II-Gq.

3) Co-expression of the receptor NK2R-Age I-EGFP and of the protein EYFP-Bgl II-Gq.

30 HEK 293 cells are transfected with the plasmids pEYFP-Bgl II-Gq or pCEP4-NK2R-AgeI-EGFP and pEYFP-Bgl II-Gq. The cells expressing the product of the genes of the plasmid pCEP4 are selected using the antibiotic hygromycin B, and those which express the products of the gene of the modified plasmid pEGFP are selected using neomycin.

35

4) Tests of functionality of the fluorescent protein Gq.

These are carried out by means of functional studies of the release of calcium. The over-expression of G proteins entails an increase in the amplitude of the calcium responses.

[CAS 25360-72-9], basic fuschin [Basic Red 9, CAS 569-61-9], Bordeaux R [Acid Red 17, CAS 5858-33-3] and Carmine [CAS 1390-65-4],

- or the EGFP is a fluorescence energy acceptor and the fluorescent substance is a fluorescence energy donor and is chosen from substances whose emission spectrum overlaps the excitation spectrum of EGFP, and in particular from: coumarins, fluorescamine, 6-(N-methylanilino)naphthalene, (mansyl) and derivatives thereof which, on the one hand, allow grafting, and, on the other hand, have an excitation spectrum which overlaps the emission spectrum of EGFP,

- or the fluorescent protein is ECFP and is a fluorescence energy donor and the fluorescent substance is an energy acceptor and is chosen from fluorescein and 7-nitro-2-benzoxa-1,3-diazole,

- or the fluorescent protein is ECFP and is a fluorescence energy acceptor and the fluorescent substance is an energy donor and is chosen from pyrene and coumarin or derivatives thereof which, on the one hand, allow grafting, and, on the other hand, have an excitation spectrum which overlaps the emission spectrum of ECFP.

8. Use according to one of Claims 1 to 7, in which the target protein is chosen from:

- membrane-bound receptors coupled to protein G in particular in Supplement Trends in Pharmacological Sciences, 1997 (*Receptor and ion Channel Nomenclature*),

- growth factor receptors, in particular those which are structurally linked to the insulin receptor (Yarden, Y. and Ullrich, A. 1988, Biochemistry 27:3113-3119) or to the  $\gamma$  interferon receptor (Brisco, J. *et al.* 1996, Phylos. Trans. R. Soc. Lond. B. Biol. Sci. 351:167-171 ; Ihle, J.N. 1995, Nature 377:591-594),

- ion channel receptors, in particular in Supplement Trends in Pharmacological Sciences, 1997 (*Receptor and ion Channel Nomenclature*),

- intracellular nuclear receptors, in particular those which are structurally linked to the steroid receptor (Mangelsdorf *et al.* 1995, Cell, 83:835-839 ; Wurtz, J.L. *et al.* 1996, Nature Struct. Biol. 3:206).

9. Use according to one of Claims 1 to 8, in which the target protein is chosen from membrane-bound receptors coupled to the G protein.

10. Process for detecting and quantifying non-covalent interactions between ~~the~~ target protein, in particular a receptor, and one of its ligands, characterized in that:



- cells or cell fragments are prepared containing a DNA sequence comprising <sup>A</sup>the gene coding for a fluorescent protein fused with <sup>A</sup>the gene for the target protein, the fusion between the gene for the fluorescent protein and the gene for the <sup>A</sup>above-mentioned target protein being such that the properties of the target protein, in particular of the receptor, are not modified by the presence of the fluorescent protein, namely: WHEREIN (NO R)

the interaction between the target protein, in particular the receptor, and the ligand is not modified. AND WHEREIN A

the response transduction function is not modified, the fluorescent protein being chosen from the fluorescent proteins obtained or derived from autofluorescent proteins of cnidarians, the molar extinction coefficient of which is greater than about  $14,000 \text{ M}^{-1} \text{ cm}^{-1}$  and the quantic fluorescence yield is greater than about 0.38, this protein being <sup>FURTHER</sup> chosen in particular from:

green fluorescent protein (GFP), or <sup>A</sup>VARIANTS OF GFP

variants derived from GFP by addition, deletion or substitution of one or more amino acids, with the proviso that these variants conserve the fluorescence property, AND

AND SAID VARIANTS

or fragments of GFP or fragments of the above-mentioned variants, with the proviso that these fragments conserve the fluorescence property.

- <sup>SAID</sup>the above-mentioned cells or <sup>SAID</sup>the above-mentioned cell fragments are placed in contact with a ligand for <sup>SAID</sup>the above-mentioned target protein, in particular for the above-mentioned receptor, <sup>SAID</sup>labeled with a label <sup>SAID</sup>consisting: COMPRISING

either of a molecule capable of absorbing the light emitted by the fluorescent protein,

or of a fluorescent substance,

and either the fluorescent protein being the fluorescence energy donor and the label being the fluorescence energy acceptor, or the fluorescent protein being the fluorescence energy acceptor and the label being a fluorescent substance which is a fluorescence energy donor, and

- irradiation is carried out at a wavelength which makes it possible either to excite the fluorescent protein or to excite the fluorescent substance,

- it being possible for the above-mentioned steps of placing in contact and irradiation to be carried out either simultaneously or one after the other, or

- <sup>SAID</sup>the above-mentioned cells or <sup>SAID</sup>the above-mentioned cell fragments are placed in contact with a ligand for the above-mentioned protein, in particular for the above-mentioned receptor, labeled with a label, the cells or the ligand having been irradiated before being placed in contact,

- either <sup>WHEREIN</sup> a reduction in the amplitude of the donor's emission and/or emission signal characteristic of the acceptor's emission is detected.

derivatives thereof which, on the one hand, allow grafting, and, on the other hand, have an excitation spectrum which overlaps the emission spectrum of EGFP,

5 and, when the label is not a fluorescent substance, it is chosen from the Acid Violet group [Acid Violet 5, CAS 10130-48-0 ; Acid Violet 7, CAS 4321-69-1 ; Acid Violet 17, CAS 4129-84-4], the Acid Red group [Acid Red 1, CAS 3734-67-6 ; Acid Red 8, CAS 4787-93-3 ; Acid Red 37, CAS 6360-07-2 ; Acid Red 40, CAS 12167-45-2 ; Acid Red 106, CAS 6844-74-2 ; Acid Red 114, CAS 6459-94-5], alizarins, aluminon, azocarmine B [CAS 25360-72-9], basic fuchsin [Basic Red 9, CAS 569-61-9], Bordeaux R [Acid Red 17, CAS 5858-33-3] and Carmine [CAS 1390-65-4],

15 - or the EGFP is a fluorescence energy acceptor and the fluorescent substance is a fluorescence energy donor and is chosen from substances whose emission spectrum overlaps the excitation spectrum of EGFP, and in particular from: coumarins, fluorescamine, 6-(N-methylanilino)naphthalene, (mansyl) and derivatives thereof which, on the one hand, allow grafting, and, on the other hand, have an excitation spectrum which overlaps the emission spectrum of EGFP.

20 14. Process according to one of Claims 10 to 13, in which the protein whose protein-ligand interaction it is desired to determine is chosen from:

- membrane-bound proteins coupled to the G protein, [in particular in Supplement Trends in Pharmacological Sciences, 1997 (Receptor and ion Channel Nomenclature),]

25 - growth factor receptors [in particular those] which are structurally linked to the insulin receptor [Yarden, Y. and Ullrich, A. 1988, Biochemistry 27:3113-3119) or to the  $\gamma$  interferon receptor (Brisco, J. *et al.* 1996, Phyl. Trans. R. Soc. Lond. B. Biol. Sci. 351:167-171 ; Ihle, J.N. 1995, Nature 377:591-594],

30 - ion channel-receptors, [in particular in Supplement Trends in Pharmacological Sciences, 1997 (Receptor and ion Channel Nomenclature),]

- intracellular nuclear receptors [in particular those] which are structurally linked to the steroid receptor [Mangelsdorf *et al.* 1995, Cell, 83:835-839; Wurtz, J.L. *et al.* 1996, Nature Struct. Biol. 3:206].

35 15. Process according to one of Claims 10 to 14, in which the fluorescent protein is EGFP and the labeled substance is Bodipy and in which either the reduction in the emission amplitude of EGFP or the emission signal of Bodipy resulting from the energy transfer is detected, the irradiation wavelength corresponding to the excitation wavelength of EGFP.

\* when the target protein is the rat glucocorticoid receptor fused at the N-terminal with, successively, a purification sequence comprising 6 histidines, a haemagglutinin epitope and a fluorescent protein and is expressed in the cell line 1471.1, the fluorescent protein is other than GFP (768 base pairs of plasmid TU65 with the mutation S65T),

\* when the target protein is the human glucocorticoid receptor truncated of its first 131 amino acids, fused at the C-terminal of a fluorescent protein in the sites Sal I and BamHI and is expressed in the cells Cos-1, the said fluorescent protein is other than that GFP as described in the article by Inouye S. and Tsuji, F. I., 1994, Febs Letters, 341:277-280,

\* when the target protein is the rat NMDA R1 sub-unit expressed in HEK 293 cells fused at the C-terminal with a fluorescent protein, the fluorescent protein is other than that consisting of the amino acids 2-238 of wild-type GFP,

\* when the target protein is a receptor or a fragment of a receptor for intracellular second messengers, the fluorescent protein is other than that GFP and its derivatives.

32. Kit or equipment for detecting and quantifying non-covalent interactions between a target protein labeled with a fluorescent protein and one of its ligands labeled with a label consisting:

- [either of] a molecule which is capable of absorbing the light emitted by the fluorescent protein,

- or [of] a fluorescent substance,

this fluorescent protein being chosen from the fluorescent proteins obtained or derived from autofluorescent proteins of cnidarians, the molecular extinction coefficient of which is greater than about  $14,000 \text{ M}^{-1}\text{cm}^{-1}$  and the quantic fluorescence yield of which is greater than about 0.38, this protein being chosen [in particular] from [ ] <sup>FURTHER</sup>

[ ] green fluorescent protein (GFP), [or] <sup>WARIANTS OF GFP</sup> variants derived from GFP by addition, deletion or substitution of one or more amino acids, with the proviso that [these variants] conserve the fluorescence property, AND

[ ] or fragments of GFP [or fragments of the above-mentioned] <sup>AND SAID</sup> variants, [with the proviso] that [these fragments] conserve the fluorescence property and its ligand labeled with a fluorescent substance, the said kit comprising:

- the target protein fused with a fluorescent protein or a stable cell line which is capable of expressing the protein fused with a fluorescent protein or a

plasmid containing the nucleic acid sequence coding for the said targets protein fused with a fluorescent protein as defined above:

- the ligand labeled with the above-mentioned label,
- the buffers and media required for the energy transfer between the above-mentioned protein and the above-mentioned ligand.

33. Kit or equipment for detecting and quantifying non-covalent interactions between a target protein labeled with a fluorescent protein (No 1) and one of its ligands labeled with a fluorescent substance corresponding to a fluorescent protein (No 2), the fluorescent protein (No 1) being chosen from the fluorescent protein EYFP or EGFP and the ligand being labeled with a fluorescent protein (No 2) ECFP, or the fluorescent protein (No 1) being ECFP and the ligand being labeled with the fluorescent protein (No 2) EYFP or EGFP, the said kit comprising:

- either a plasmid containing a nucleic acid sequence coding for the target protein fused with a fluorescent protein (No 1), and
  - \* a plasmid containing a nucleic acid sequence coding for the ligand fused with a fluorescent protein (No 2), or
  - \* a ligand fused with a fluorescent protein (No 2) obtained via a recombinant route and purified, or
- or a stable cell line which is capable of expressing the target protein fused with a fluorescent protein (No 1), and
  - \* a stable cell line which is capable of expressing the ligand fused with a fluorescent protein (No 2) or
  - \* a ligand fused with a fluorescent protein (No 2) obtained via a recombinant route and purified, AND
- the buffers and media required for the energy transfer between the above-mentioned protein and the above-mentioned ligand.

34. Kit or equipment for detecting and quantifying non-covalent interactions between a target protein consisting of a receptor coupled to the G protein labeled with a fluorescent protein (No 1) and the G protein labeled with a fluorescent substance corresponding to a fluorescent protein (No 2), the fluorescent protein (No 1) being chosen from the fluorescent protein EYFP or EGFP and the G protein being labeled with the fluorescent protein (No 2) ECFP or the fluorescent protein (No 1) being ECFP and the G protein being labeled with the fluorescent protein (No 2) EYFP or EGFP, the said kit comprising:

- either a plasmid containing a nucleic acid sequence coding for the receptor fused with a fluorescent protein (No 1), and

(NO 2)

- ☒ a plasmid containing a nucleic acid sequence coding for the G protein fused with a fluorescent protein (No 2), or
- ☒ the G protein fused with a fluorescent protein (No 2), obtained via a recombinant route and purified; <sup>OR</sup>
- 5 - ☒ a stable cell line which is capable of expressing the receptor fused with a fluorescent protein (No 1), and
- ☒ a stable cell line which is capable of expressing the G protein fused with a fluorescent protein (No 2), or,
- ☒ the G protein fused with a fluorescent protein (No 2), obtained via a recombinant route and purified; <sup>AND</sup>
- 10 - ☒ the buffers and media required for the energy transfer between the above-mentioned receptor and the above-mentioned G protein.